SILENCING STMN1 EXPRESSION DECREASES AGGRESSIVE PHENOTYPE IN ADRENOCORTICAL CARCINOMA

A Thesis

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Master of Science

by

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ABSTRACT

Background: Adrenocortical carcinoma (ACC) is a rare endocrine malignancy with a poor prognosis and few therapeutic options. Stathmin1 (STMN1) is a cytosolic protein involved in microtubule dynamics, which has been implicated in carcinogenesis and more aggressive behavior in multiple epithelial malignancies. Here we aimed to evaluate the expression of STMN1 in ACC and to elucidate how this may contribute to its malignant phenotype.

Methods: STMN1 was identified as a highly differentially expressed gene in human samples of adrenocortical carcinoma submitted for high throughput RNA-sequencing compared to benign adrenal tumors. Expression was confirmed by qRT-PCR, Western blot, and immunohistochemical (IHC) staining of a tissue microarray (TMA) from two independent cohorts. The biologic relevance of this target was investigated in the NCI-H295R ACC cell line by lentivirus-mediated silencing of STMN1. *In vitro* phenotypic changes were assessed for cell viability, cell-cycle alterations, invasion, migration, and anchorage-independent growth.

Results: Differential gene expression demonstrated over an eight-fold increase in STMN1 mRNA in malignant compared to benign adrenal tissue ($p<2.4E-07$). IHC showed significantly higher expression of STMN1 protein in ACC compared to normal and benign tissues ($p<0.05$, $p<0.01$, respectively).

We achieved more than 70% reduction of STMN1 expression in NCI-H295R by lentivirus-mediated short hairpin RNA (shRNA) delivery. STMN1 knockdown resulted in decreased cell viability ($p<0.01$), cell-cycle arrest at G_0/G_1 ($p<0.01$), and increased apoptosis ($p<0.01$) in stressed, serum-starved conditions compared to scramble shRNA controls. STMN1 knockdown also decreased migration ($p<0.05$), invasion ($p<0.05$), and anchorage-independent growth ($p<0.01$) compared to controls.

Conclusion: STMN1 is overexpressed in human ACC samples and knockdown of this target *in vitro* resulted in a less aggressive phenotype of ACC, particularly under serum-starved conditions. Further study is needed to investigate the feasibility of interfering with STMN1 as a potential therapeutic target.

BIOGRAPHICAL SKETCH

Anna Aronova, MD grew up in St. Petersburg, Russia and immigrated to the United States in 1992 with her family. After completing high school at Staten Island Technical HS, She obtained a Bachelor's of Science degree in 2006 from Cornell University majoring in Human Biology, Health and Society. She then went on to obtain her Medical degree in 2010 from Weill Cornell Medical College, where she graduated with Honors in Research. Subsequently, she began her General Surgery Residency at New York Presbyterian Hospital in 2010. After three clinical years, she took two year off to do basic and clinical science research in the laboratory of Dr. Thomas J. Fahey, III and Dr. Rasa Zarnegar from 2013 to 2015. Since then, she has returned to clinical residency and is completing her general surgery training in June 2017. She has been accepted to the Mount Sinai School of Medicine Fellowship in Endocrine Surgery and will be starting the program in August 2017.

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INTRODUCTION

Adrenocortical carcinoma (ACC) is a rare but deadly malignancy representing 0.7-2.0 cases of carcinoma per million per year and manifesting in a 5-year overall survival of 37-47% [1–3]. Although some patients present with symptoms from hormonal hypersecretion or mass effect, detection of ACC is often incidental and at an advanced stage. En-bloc, R0 surgical resection remains the mainstay of therapy as the only potential curative option for patients with ACC [4,5]. For those unable to undergo resection or with extensive tumor burden, adrenolytic or conventional chemotherapeutic regimens yield modest results, and their use is limited by toxicity [1,6–8]. While there is much ongoing research to help understand the pathogenesis of this disease and improve outcomes, attempts at targeted therapy against vascular endothelial growth factor (VEGF) receptor thus far have failed to show survival benefit [9]. Similarly disappointing results were shown in a recent phase 3 randomized, controlled trial of lisitinib, an oral small molecule inhibitor of insulin growth factor (IGF)-1 receptor [10–12]. Consequently, it is imperative to continue the search for novel and more effective therapeutic targets for ACC.

In an effort to further understanding of the pathogenesis of this disease and to identify potential targets for treatment in ACC, we performed RNA-sequencing of a set of 38 human adrenal samples comprised of normal adrenal cortex (NML), adrenal adenomas (AA) and adrenocortical carcinomas

(ACC). Given the recent unsuccessful small-molecule inhibitor trials against transmembrane receptors, we focused our attention on the differential expression of cytosolic proteins in the malignant compared to benign samples. Thereby, we identified STMN1 to be among the most highly differentially expressed genes.

Stathmin 1 (STMN1, also known as p17, p18, p19, LAP 18, metablastin, and oncoprotein 18) is a 19kD cytosolic phosphoprotein, involved in the regulation of microtubule dynamics by inhibition of tubulin polymerization and promotion of microtubule depolymerization [13]. Its role in carcinogenesis has been investigated in a variety of tumor types and its expression has been associated with tumor progression and poor prognosis in breast, gastric, esophageal, endometrial, hepatocellular, oral squamous cell, and colorectal cancers [14–20]. Furthermore, STMN1 has been exploited to understand its role in oncogenesis and for examining its therapeutic potential. In colorectal cancer, silencing STMN1 inhibited colorectal cancer metastases by downregulating transcription of metastatic drivers and enhanced tumor chemoresponse to 5-fluorouracil [21]. In neuroblastoma, STMN1 suppression reduced lung metastases in a mouse model [22]. Wang et al. also showed that STMN1 knockdown improved sensitivity to tubulin-targeting drugs paclitaxel and vinblastine in esophageal squamous cell carcinoma [23]. Despite this growing body of literature on the importance of STMN1 in the pathogenesis of a number of epithelial malignancies, its role in adrenocortical carcinoma has not yet been elucidated.

Herein, we report on the expression of STMN1 in ACC and demonstrate its role in the phenotype of ACC *in vitro.* This study provides a potential stepping-stone from which to pursue targeted therapy against this highly heterogeneous and aggressive malignancy.

MATERIALS AND METHODS

Tissue Specimens

After obtaining approval from the Weill Cornell Medical College Institutional Review Board, adrenalectomy specimens from patients undergoing surgery at Weill Cornell Medical College/New York Presbyterian Hospital from June 2000 to September 2011 were identified using a prospectively maintained tissue database. Samples were collected after written informed consent was obtained from each patient. Tissues were snap-frozen in liquid nitrogen at the time of surgery and stored at -80°C. In addition, 5 tumor samples were obtained from the Cooperative Human Tissue Network, funded by the National Cancer Institute (NCI). Thirteen ACC, 14 AA, and 11 NML samples were chosen for further analysis. An endocrine pathologist (TS) reviewed each case to ensure correct diagnosis.

RNA Extraction and Next-Generation RNA Sequencing

RNA was extracted from 38 frozen tissue samples and cells using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. RNA quality was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and RNA quantity was assessed with the NanoDrop (Thermo Fischer Scientific, Wilmington, DE). An RNA Integrity Number (RIN) of ≥8 was required for library preparation. Samples were prepared for Next-Generation sequencing with the TruSeq RNA sample prep

kit (Illumina, San Diego, CA). Paired-end cluster generation was performed utilizing the TruSeq SBS Kit v3-Hs in conjunction with the TruSeq PE Cluster Kit v3-cBot-HS on the HiSeq2000 (Illumina, San Diego, CA). Illumina HiSeq control software performed a real-time analysis of the sequencing runs. Three samples were run in each lane using paired end mode (2 x 51 cycles).

A median of 1.2 $x10^8$ reads (range 9.2 $x10^7$ and 1.63 $x10^8$ reads) was obtained per sample. The mean perfect index reads percentage was 87.7±3.5%. These values were within the parameters suggested by Illumina. Reads were aligned to the human genome (GRCH37/hg19) using TopHat [24,25] (http://tophat.cbcb.umd.edu/) with default parameters and mRNA levels quantified using CuffLinks [26] (http://cufflinks.cbcb.umd.edu/) with upper-quartile and GC normalization. Differentially expressed genes were identified using the LIMMA approach comparing ACC (malignant) v NML+AA (benign), and ranked by false discovery rate (FDR) and absolute log fold change >2. An FDR of ≤0.05 was considered significant. This yielded several hundred differentially expressed genes. To narrow this, we restricted our search to cytosolic proteins using the Gene Ontology database GO:005829 annotation.

Immunohistochemistry Staining and Grading

The Cornell tissue microarray (TMA) was constructed from formalin-fixed paraffin embedded (FFPE) tissue blocks from the same 38 patients submitted for sequencing, with an additional 14 ACC samples only available as FFPE blocks. Three cores of 0.6mm were taken from areas of high tumor

density as identified by TS on corresponding hematoxylin and eosin (H&E) stained slides. Each case was represented by triplicate cores.

Immunohistochemical staining of STMN1 (Rabbit Polyclonal, Dilution at 1:50, Cell Signaling) was accomplished using the Bond III Autostainer (Leica Microsystems, Buffalo Grove, Illinois). Formalin-fixed, paraffin-embedded tissue sections were first baked and deparaffinized. Antigen retrieval was followed using the Bond Epitope Retrieval Solution 2(ER2) at 99-100°C for 20 minutes (Leica Microsystems, Buffalo Grove, Illinois). Sections were then subjected to sequential incubations with primary antibody, post-primary, polymer, endogenous peroxidase block, diaminobenzidine (DAB) and hematoxylin for 15, 8, 8, 5, 10 and 5 minutes (Bond Polymer Refine Detection; Leica Microsystems), respectively. Finally, stained sections were dehydrated and mounted in Cytoseal^{TM} XYL (Richard-Allan Scientific, Kalamazoo, MI).

Grading of STMN1 staining was performed by an endocrine pathologist (TS) using a semi-quantitative scoring system. A STMN1 score of 0, 1+, 2+, or 3+ was considered to represent no staining, weak staining, moderate staining, or strong staining, respectively. Appropriate positive and negative (incubation with secondary antibody only) controls were stained in parallel with each round of immunohistochemistry.

A validation TMA from MD Anderson was used for confirmation of expression findings. This cohort consisted of 39 ACCs, 19 AAs, and 19 NML

samples. STMN1 staining was performed and analyzed independently by pathologist SG.

Reverse Transcription and Quantitative PCR

First-strand cDNA synthesis was performed using M-MLV Reverse Transcriptase (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The quantitative PCR (qPCR) was performed with the TaqMan® Gene Expression Assay (Life Technologies, Carlsbad, CA) using predesigned primers for STMN1 *(*Hs01027515_gH) and *GAPDH (*Hs02758991_g1) according to the manufacturer's instructions using the Lightcycler 480 II (Roche Diagnostics, Indianopolis, IN). All PCR reactions were performed in a final volume of 10uL with 1uL of cDNA template. The following thermal cycling parameters were used: incubation at 50°C for two minutes, denaturing at 95°C for ten minutes, 45 cycles of the amplification step (denaturation at 95°C for 15 seconds and annealing/extension at 60°C for one minute). STMN1 gene expression was normalized relative to the housekeeping gene GAPDH. All experiments were performed in triplicate and gene expression values were calculated according to the $\Delta\Delta$CT method [27], using cDNA derived from NCI-H295R cells as the reference.

Protein Preparation and Western Blot Analysis

Protein was extracted with RIPA-Lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA). Protein concentration was determined by the

Pierce BCA assay method according to the manufacturer's protocol (Thermo Scientific, Waltham, MA). Western blots were performed with 10 micrograms of protein per lane using semi-dry transfer and horseradish-peroxidase (HRP)-conjugated antigen retrieval system as described previously [28]. STMN1 antibody was obtained from Cell Signaling (Danvers, MA) and it was normalized to GAPDH or Actin (Cell Signaling, Danvers, MA). Quantification of relative densities was completed using ImageJ software (NIH).

Cell Culture

The adrenocortical carcinoma cell line NCI-H295R (ATCC, Rockville, MD) is an angiotensin II-responsive steroid-producing line isolated from a 48-year old female patient with ACC in 1980 [29]. It is the most widely used commercially available ACC cell line. The cells were grown and maintained in DMEM/F12 supplemented with 1% ITS+ Premix (BD Biosciences, San Jose, CA), 2.5% NuSerum I (BD Biosciences), and 1% Penicillin/Streptomycin/Amphotericin (P/S/A) in a standard humidified incubator at 37°C in a 5% CO_2 atmosphere. Cells were routinely subcultured and medium was changed every 2-3 days. For particular experiments, serum free media was utilized for specific durations consisting of DMEM/F12 supplemented with 1% P/S/A only.

Lentivirus-mediated Short Hairpin RNA (shRNA) Knockdown of Gene Expression

To knock down STMN1 expression, OP18 shRNA transduction-ready lentiviral particles containing three STMN1-specific constructs added in equal ratios were purchased (SantaCruz Biotechnology Inc, Santa Cruz, CA). The hairpin sequences were: 5'-GATCCCCAGATCCAGACTGTAAGATTCAAGAGATCTTACAGTCTGGATC TGG TTTTT-3', 5'-GATCCGGGAGAAACTGAAAGTGTTTTCAAGAGAAACACT TTCAG TTTCTCCCTTTTT-3', and 5'-GATCCCCTCCTGGTTGATACTTGTTTCA AGAGAACAAGTATCAACCAGGAGGTTTTT-3'. Transduction was carried out according to the manufacturer's instructions. Briefly, $1x10^5$ H295R cells were seeded in complete media in one well in a 24-well plate. After 48 hours, media was changed to complete media with 5 ug/mL Polybrene (Santacruz Biotechnology Inc, Santa Cruz, CA) and $5x10^5$ lentiviral particles were added. 24 hours post-infection, Polybrene-containing media was removed and replaced with regular complete media. Cells were split 1:3 once appropriate and stable clones expressing the shRNA were selected by Puromycin (Santacruz Biotechnology Inc, Santa Cruz, CA) selection at 5ug/mL, as predetermined via a kill curve on uninfected cells. The same protocol was simultaneously carried out for control shRNA (Santacruz Biotechnology Inc, Santa Cruz, CA) containing a construct encoding a non-targeting scramble sequence. Once stable clones were selected, cells were maintained in Puromycin-containing complete media. Successful knockdown of STMN1 expression was assayed by qPCR and Western blot as described above.

Cell Proliferation Assay

The Vybrant MTT Cell Proliferation Assay Kit (Life Technologies, Carlsbad, CA) was used to assess cell proliferation. 30,000 viable cells were seeded per well in a 96-well plate in a final volume of 100uL of phenol free complete media and phenol-free serum-free media. Every 24 hours, media was replaced and a plate was subjected to the assay by adding 10uL of 12mM MTT stock solution, as per manufacturer's instructions. In brief, the plate was incubated at 37°C for 4 hours, after which point all but 25uL was removed and 50uL of dimethyl sulphoxide (DMSO) (Sigma Aldrich, St. Louis, MO) was added to each well. The plate was incubated at 37°C for an additional 10 minutes and absorbance was read in a microplate reader (iMARK, BioRad, Hercules, CA) at 490nm. Experiments were performed twice in quintuplet.

Cell Cycle and Apoptosis Analysis

For cell cycle analysis, $5x10^5$ cells were seeded in 6-well plates overnight. Media was replaced with serum-free media in a subset of wells the following morning and cells were harvested and fixed after 48 hours of serum starvation by utilizing cold PBS and 70% cold ethanol, respectively. After fixation at 4°C for 2 days, cells were washed twice with PBS, treated with 100ug/mL RNaseA for 30 minutes at 4°C, washed once with PBS, and stained with 50ug/mL propidium iodide (PI). The cells were analyzed by flow cytometry (Gallios, Beckman Coulter, Brea, CA) and cell-cycle stages were determined

using the Kaluza software (Beckman Coulter, Brea, CA). 20,000 events per sample were analyzed. For apoptosis analyses, the PE Annexin V Apoptosis Detection Kit I was used (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Similarly, a subset of cells were serum-starved for 48 hours before the cells and supernatant was harvested, washed with PBS, resuspended in 1x Binding Buffer. The cells were then stained with AnnexinV and 7AAD, incubated at room temperature for 15 minutes in the dark and analyzed by flow cytometry as above. The experiments were performed twice in sextuplet.

Cell Migration Assay

A scratch assay was used to assess for cell migration *in vitro.* Cells were seeded in 6 well plates and grown to ~60-80% confluence. A 200uL pipette tip was used to create a horizontal scratch across the plate at three locations. Complete media was changed every 3 days. Cells were photographed daily and percent migration was measured in 6 fields using ImageJ software. The experiment was performed twice.

Cell Invasion Assay

The extent of cell invasion was assessed with the Cytoselect 24-well Cell Invasion Assay (Cell Biolabs Inc, San Diego, CA) according to the manufacturer's instructions. A total of $2x10^5$ cells in serum free media were seeded onto inserts (8uM pore-sized pre-coated extracellular basement

membrane). The inserts were placed in a 24-well plate containing complete media. The plates were incubated for 48h at 37°C. Cells that invaded the matrix to the lower surface of the membrane were stained and counted under a light microscope. Four fields in 4 separate quadrants of each membrane were counted and averaged. The experiment was performed twice.

Soft Agar Anchorage-Independent Growth Assay

Two-layered soft agar assays were performed in 6-well plates. The bottom layer of agar (1ml/well) contained 0.5% agar (Sigma Aldrich, St. Louis, MO) in maintenance medium. 50,000 cells in maintenance medium were mixed with agarose for a 0.35% solution and allowed to solidify atop the agar layer. This was then overlaid with 2mL complete media and allowed to grow at 37°C in 5% CO_2. Media was changed twice per week. After 3 weeks of culture, cell colonies were stained with 0.005% crystal violet solution and examined by microscopy. Colonies were counted in four separate fields per well and summed for each of 6 replicates. The experiment was performed twice.

Statistical Analysis

Calculations to determine significance in all experiments except RNA sequencing analyses (for which the methods are described above) were carried out using Pearson's chi-squared test, student's T-test, Mann-Whitney U-test, one-way Analysis of Variance, or Kruskal-Wallis test, as appropriate.

Continuous variables that followed a normal distribution are presented as mean ± standard deviation (SD), while those that were not normally distributed are presented as median (range). A *p*-value of less than 0.05 was considered statistically significant. All statistical analyses (except those performed for RNA sequencing analysis, as described above) were performed using STATA version 13.0 (College Station, TX).

RESULTS

STMN1 expression is markedly increased in ACC compared to AA and NML

RNA-sequencing analyses identified >300 genes that met our inclusion criteria for highly differentially expressed cytosolic proteins as described in the methods section. Figure 1A represents the top 20 most differentially expressed genes between benign (AA+NML) and malignant samples (ACC). STMN1 was among the top differentially expressed genes in ACC, with more than an 8-fold increase of STMN1 expression in malignant over benign adrenal tissue ($p<2.4E-07$) (Figure 1B). The expression of STMN1 was validated by qPCR and Western Blot (Figure 2A,B), which demonstrated distinctly higher expression of STMN1 in ACC samples and established ACC cell line, NCI-H295R, compared to normal adrenal cortex and adrenal adenoma samples.

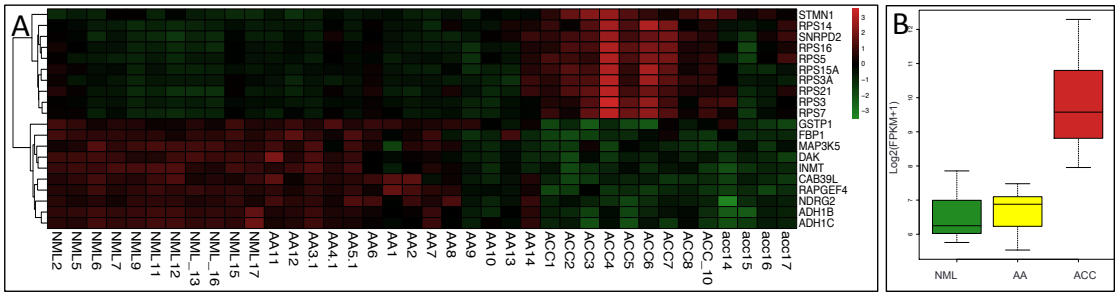

Figure 1. (A) Heatmap of unsupervised clustering results from RNA sequencing of 11 Normal Adrenals (NML), 14 Adrenal Adenomas (AA), 13 Adrenocortical Carcinomas (ACC) depicting the top 20 most differentially-expressed genes. **(B)** Quantitative expression of STMN1 in ACC compared to AA and NML.

We further investigated protein expression using IHC on a tissue microarray composed of 51 samples and found significantly higher STMN1 expression compared to NML and AA (Figure 2C-F). Wide distribution of

STMN1 was detected in the majority of tumor cells in ACC. To confirm our findings, we compared IHC staining of STMN1 on an independent TMA (MDA TMA) and found a stronger staining pattern overall and a similarly high expression of STMN1 in ACC samples relative to both NML and AA (Figure 2G). Available clinical characteristics of patients represented by both TMAs, including age, sex, biochemical profile, presence of metastases, histopathologic features (e.g. Weiss score), and length of followup, were not significantly different (data not shown).

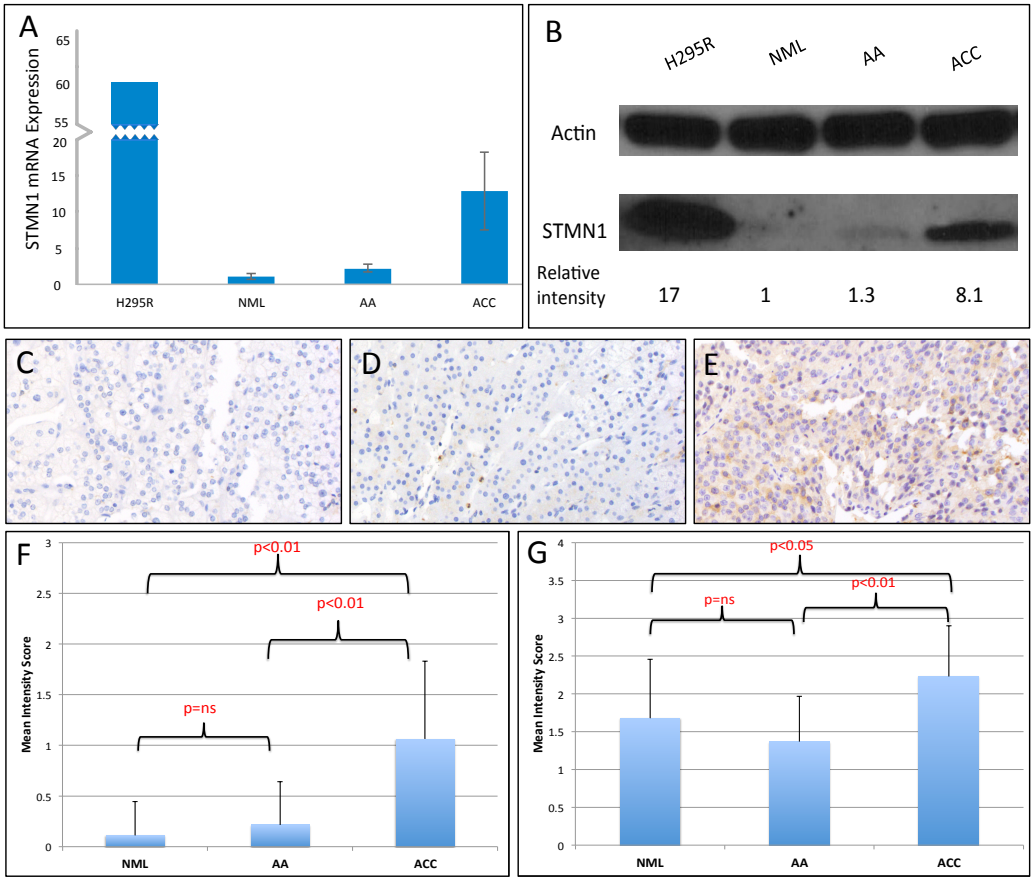

Figure 2. (A) Relative STMN1 expression in adrenocortical tissue and cell lines. mRNA expression of STMN1 was normalized to GAPDH using the ($2^{-\Delta\Delta CT}$) method. Values are relative to NML. **(B)** Representative expression of STMN1 protein by Western Blot. **(C-E)** Representative expression of STMN1 protein by Immunohistochemistry in NML (C), AA (D), and ACC (E). **(F)** Immunohistochemical staining of Cornell TMA for STMN1 (NML: n=10, AA: n=14, ACC: n= 27). **(G)** Immunohistochemical staining of MDA TMA for STMN1 (NML: n=19, AA: n=19, ACC: n= 39). ANOVA statistical test was used for comparisons between groups.

Effect of STMN1 knockdown on cellular proliferation, cell cycle and apoptosis in ACC cell line

Given the high expression of STMN1 in ACC, we next investigated the phenotypic effects of STMN1 knockdown in adrenocortical carcinoma cells using the NCI-H295R cell line to investigate STMN1's ACC oncogenic potential. Stable knockdown of STMN1 was achieved via lentiviral-assisted shRNA transfection using puromycin selection. We achieved a 70% knockdown of SMTN1 expression at the mRNA level and a 78% knockdown of protein expression compared to control cells transfected with a scrambled sequence (Figure 3A,B).

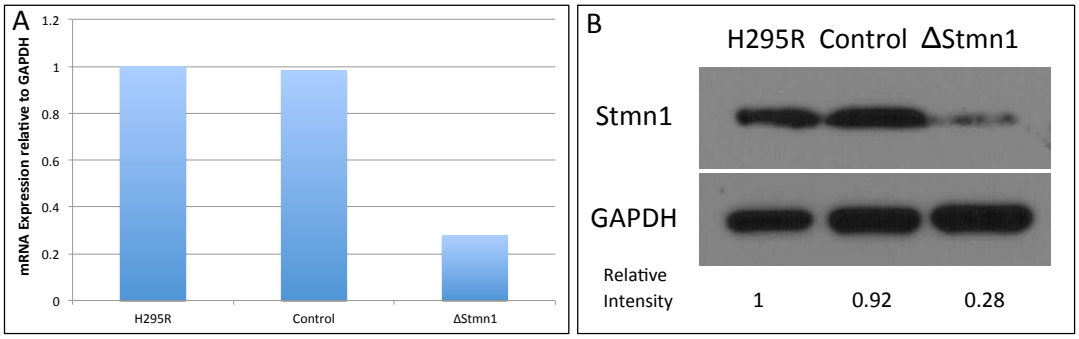

Figure 3. Lentiviral-assisted stable knockdown of STMN1 in NCI-H295R cells. **(A)** Expression of STMN1 mRNA without transfection (H295R) and after transfection with scramble shRNA (Control) and STMN1-shRNA (ΔStmn1) normalized to GAPDH using the ($2^{-\Delta\Delta CT}$) method. **(B)** Representative Western blot demonstrating decreased protein expression of knockdown cells (ΔStmn1) compared to untreated cells (H295R) and cells treated with scramble shRNA (Control).

The effect of STMN1 knockdown on cell viability was investigated using the MTT assay. No significant difference was seen between control and STMN1 knockdown NCI-H295R cells (ΔSTMN1)(Figure 4A). Next, to simulate a tumor microenvironment, we cultured these cells in serum-free conditions. Cell viability was modestly reduced in knockdown cells compared to control

when serum was removed, $p<0.01$ (Figure 4B). This effect was seen starting at 24 hours. Cell cycle analysis (Figure 4C) demonstrated a greater proportion of cells remaining in G0/G1 under stressed conditions (76.9% vs. 73.5%, $p<0.01$ in ΔSTMN1 vs. control, respectively) compared to regular growth media (68.7% vs. 66.2%, $p=0.09$ in ΔSTMN1 vs. control, respectively). Assessing apoptotic processes, we found a significantly greater proportion of viable cells (41.5% vs. 13.4%, $p<0.01$) and fewer apoptotic cells (39.6% vs. 73.3%, $p<0.01$) in the control group compared with the knockdown under serum-free conditions (Figure 4D). No significant difference was found in cell cycle or apoptosis between the STMN1-infected cells and control cells in regular serum-containing media. These results indicate that under stressed conditions, the loss of STMN1 induced apoptosis and cell-cycle arrest at G0/G1.

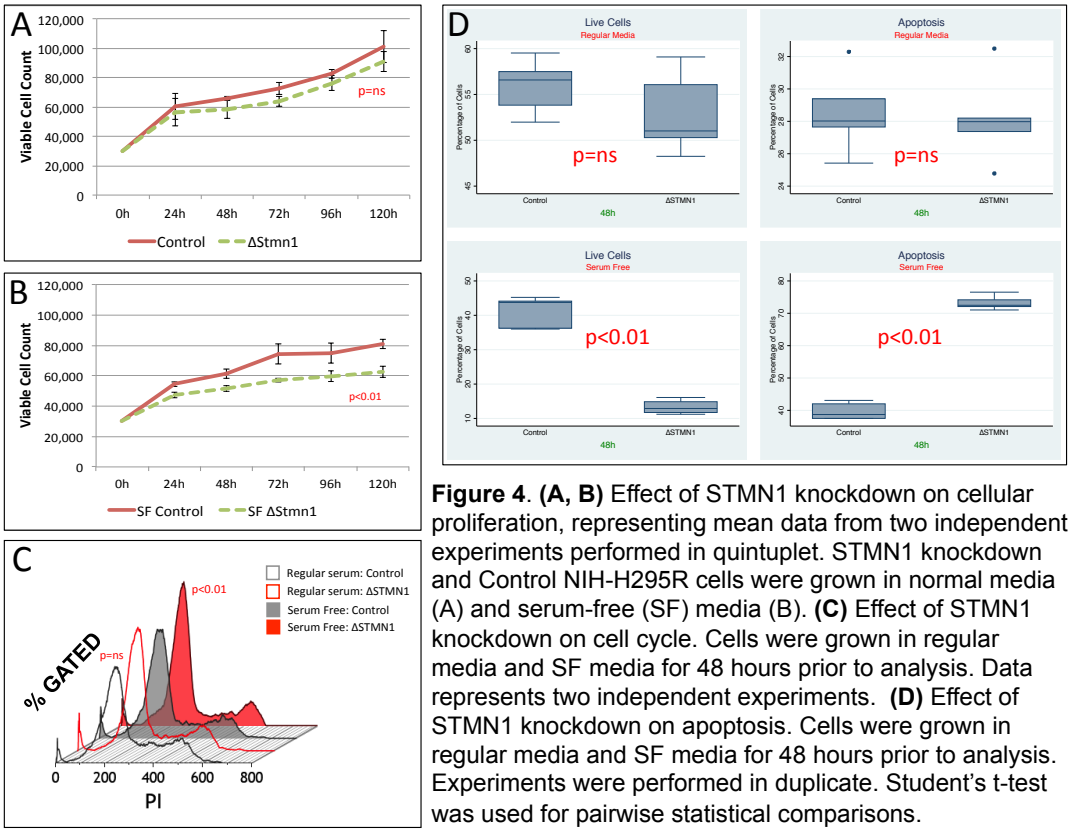

Figure 4. **(A, B)** Effect of STMN1 knockdown on cellular proliferation, representing mean data from two independent experiments performed in quintuplet. STMN1 knockdown and Control NIH-H295R cells were grown in normal media (A) and serum-free (SF) media (B). **(C)** Effect of STMN1 knockdown on cell cycle. Cells were grown in regular media and SF media for 48 hours prior to analysis. Data represents two independent experiments. **(D)** Effect of STMN1 knockdown on apoptosis. Cells were grown in regular media and SF media for 48 hours prior to analysis. Experiments were performed in duplicate. Student's t-test was used for pairwise statistical comparisons.

Effect of STMN1 knockdown on cell migration and invasion

To investigate STMN1's role in cellular motility and invasive potential, we subjected transfected cells to a scratch assay for 5 days. ΔSTMN1 cells were found to migrate 11% less than control cells transfected with scramble shRNA ($p<0.05$) as shown in Figure 5A, B. Cellular invasion was assessed using a Boyden Chamber assay after 48 hours of incubation. Cells transfected with STMN1-shRNA demonstrated less invasive ability than control cells transfected with scramble-shRNA ($p<0.05$), Figure 5C.

STMN1 knockdown decreases tumorogenicity in NCI-H295R

To further evaluate the phenotypic changes incurred by STMN1 knockdown, we performed the soft-agar anchorage-independent growth assay. ΔSTMN1 cells formed significantly fewer colonies in soft agar after three weeks of incubation compared to control (Figure 5D,E; 837 vs. 637 colonies, $p<0.01$) suggesting STMN1 contributes to tumorigenesis.

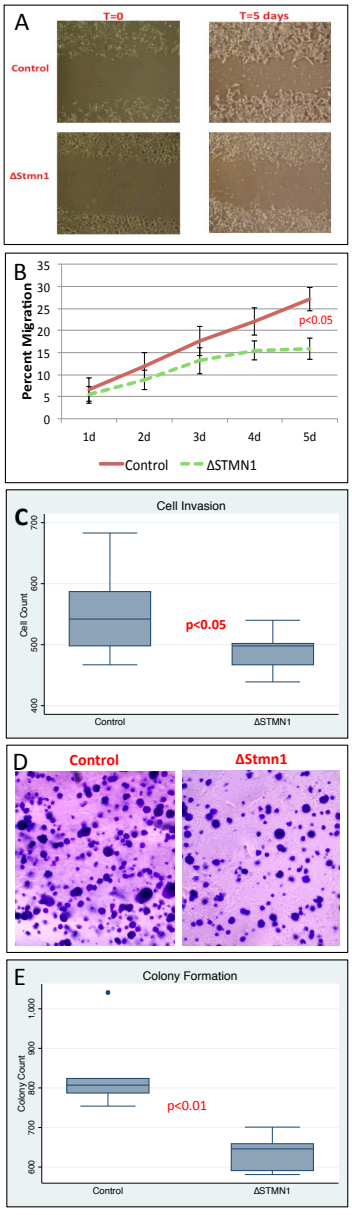

Figure 5. (A) Representative images of cell migration at Time=0 and T=5 days in control and STMN1 knockdown cells. **(B)** Quantitative assessment of cell migration from two independent experiments. **(C)** Effect of STMN1 knockdown on cell invasion showing significantly decreased invasion ability of STMN1 knockdown cells. Data representing two independent experiments. **(D)** Representative images of reduced anchorage-dependent growth in soft agar with the loss of STMN1, showing colony formation after 3 weeks of incubation. **(E)** Quantitative assessment of colony formation. Data representing two independent experiments. Student's t-test was used for pairwise statistical comparisons in B, C, and E.

DISCUSSION

STMN1 is a cytosolic protein that plays an important regulatory role in microtubule dynamics. It was originally described as being overexpressed in leukemia and lymphoma [30], and has since been described in multiple solid organ human malignancies where it is correlated with aggressive cellular behavior as well as poor clinicopathologic and prognostic features [14,16,17,19,31]. Furthermore, investigations into inhibition of STMN1 with or without compounding strategies have shown promise for an anti-STMN1 approach as a potential therapeutic target [21–23]. We sought to understand whether STMN1 contributes to the pathogenesis of adrenal cortical carcinoma as a gateway for further investigation into its therapeutic potential.

We submitted human adrenocortical carcinoma samples from a single institution for RNA sequencing and found STMN1 to be highly overexpressed in ACC compared to benign adrenal samples. To elucidate the functional consequences of STMN1 knockdown, we employed lentiviral-mediated STMN1 shRNA in the ACC cell line NCI-H295R and evaluated proliferation, invasion, migration and colony formation. Furthermore, in order to simulate a tumor microenvironment devoid of nutrients and growth factors, we stressed cells in our experiments by removing serum from cell culture media, as previously described [32–34]. In doing so, we found significant effects on cellular viability, cell-cycle progression, apoptosis, and invasion.

These results suggest that STMN1 overexpression confers a survival advantage or escape mechanism and silencing its expression may be a mechanism to curb aggressive proliferation. While the mechanism behind this relationship is still elusive, insights into how STMN1 regulates microtubules and proliferation have been suggested by Rubin and Atweh [35]. They hypothesize that STMN1 plays a critical role in the formation of the normal mitotic spindle upon entry into mitosis but also in the regulation of function in later stages of mitosis and its timely exit from this cell-cycle process. Specifically, they report that expression of a constitutively active form of STMN1 arrests cells early in mitosis and prevents further progression through the cell cycle. Lu et al [36] suggest that the microtubule destabilizing activity of STMN1 plays a crucial role in initiating the epithelial-mesenchymal transition (EMT) by contributing to a 'stathmin-microtubule-EMT' axis during cancer development, resulting in features such as high invasiveness and drug resistance [36].

Furthermore, among other highly regulated processes essential to normal cellular function is apoptosis. Dysregulation of apoptosis has been widely implicated in the development of cancer, as malignant cells are often dependent on aberrant apoptosis to escape cell death. As such, the apoptotic pathway has been an attractive target for the development of therapeutics [37]. In this study, we found that silencing STMN1 significantly increased apoptosis in H295R cells, suggesting the potential therapeutic utility of this approach.

The role of STMN1 in the tumorigenesis of ACC is further suggested by our findings that silencing STMN1 decreased migration, invasion, and anchorage-independent growth of ACC tumor cells. Similar findings have been reported in esophageal adenocarcinoma, endometrial adenocarcinoma and oral squamous cell carcinoma, among others [15,18,19].

Finally, STMN1 overexpression has been linked to chemoresistance to taxanes and vinblastine (chemotherapeutic drugs that target microtubules) in some tumor types. In breast cancer, Alli et al. showed that overexpression of STMN1 decreased sensitivity of paclitaxel and vinblastine and suggested it did so by altering drug binding and inducing arrest of the cell cycle [38]. In esophageal cancer, Wan et al. showed that silencing STMN1 increased sensitivity to the same drugs [23]. Further, in NCI-H295R cells, Fallo et al. reported an antiproliferative effect from paclitaxel by inducing apoptosis [39]. Future studies combining STMN1 silencing with microtubule-targeting drugs are warranted.

CONCLUSION

In adrenocortical carcinoma, therapeutic options for advanced disease are lacking. With the advent of novel technologies such as genetic profiling, small molecule inhibitors and precision medicine, we are offered novel and potentially more viable options for the treatment of these rare and lethal cancers. Although further studies are needed to determine the viability of STMN1 as a therapeutic target in ACC, our study provides evidence supporting its relevance to the tumorigenesis of ACCs.

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